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## **Results of the 4th scientific workshop of the ECCO (I): Pathophysiology of intestinal fibrosis in IBD**

Latella, Giovanni ; Rogler, Gerhard ; Bamias, Giorgos ; Breynaert, Christine ; Florholmen, Jon ;  
Pellino, Gianluca ; Reif, Shimon ; Specia, Silvia ; Lawrance, Ian C

**Abstract:** The fourth scientific workshop of the European Crohn's and Colitis Organization (ECCO) focused on the relevance of intestinal fibrosis in the disease course of inflammatory bowel disease (IBD). The objective was to better understand the pathophysiological mechanisms of intestinal fibrosis, to identify useful markers and imaging modalities of fibrosis in order to assess its presence and progression, and, finally, to point out possible approaches for the prevention and the treatment of fibrosis. The results of this workshop are presented in three separate manuscripts. This first section describes the most important mechanisms that contribute to the initiation and progression of intestinal fibrosis in IBD including the cellular and molecular mediators, the extracellular matrix molecules and matrix metalloproteinases/tissue inhibitors of metalloproteinases-system, the microbiota products, the role of fat, genetic and epigenetic factors, as well as the currently available experimental models. Furthermore, it identifies unanswered questions in the field of intestinal fibrosis and provides a framework for future research.

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## **Results of the 4th Scientific Workshop of the ECCO (I): Pathophysiology of Intestinal Fibrosis in IBD**

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## **ABSTRACT**

The fourth scientific workshop of the European Crohn's and Colitis Organization (ECCO) focused on the relevance of intestinal fibrosis in the disease course of inflammatory bowel disease (IBD). The objective was to better understand the pathophysiological mechanisms of intestinal fibrosis, to identify useful markers and imaging modalities of fibrosis in order to assess its presence and progression, and, finally, to point out possible approaches for the prevention and the treatment of fibrosis.

The results of this workshop are presented in three separate manuscripts. This first section describes the most important mechanisms that contribute to the initiation and progression of intestinal fibrosis in IBD including the cellular and molecular mediators, the extracellular matrix molecules and matrix metalloproteinases/tissue inhibitors of metalloproteinases-system, the microbiota products, the role of fat, genetic and epigenetic factors, as well as the currently available experimental models. Furthermore, it identifies unanswered questions in the field of intestinal fibrosis and provides a framework for future research.

**KEYWORDS:** Inflammatory bowel disease, ulcerative colitis, Crohn's disease, intestinal fibrosis, extracellular matrix, matrix metalloproteinases, tissue inhibitors of metalloproteinases, microbiota, mesenteric adipose tissue, genetics, epigenetics, animal models.

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## 1. INTRODUCTION

Fibrogenesis is a “physiological process” triggered by inflammation that may lead to tissue repair or fibrosis depending on the balance between production and degradation of extracellular matrix(ECM) proteins(1). Fibrosis occurs when regeneration and repair fail to restore normal tissue architecture and can lead to organ malfunction and death. Although fibrosis is increasingly recognized as a problem, there are few, if any, treatment strategies available. Fibrosis is a common problem in inflammatory bowel disease(IBD) but which factors trigger chronicity and promote fibrosis is not known(2,3).

Inflammation is necessary for fibrosis, but it subsequently plays a minor role in its progression and anti-inflammatory treatment in IBD may not prevent fibrosis once excessive ECM deposition has started(4). Mechanisms that regulate fibrosis, therefore, appear to be distinct from those regulating inflammation. Fibrosis, however, follows the distribution of inflammation. In ulcerative colitis (UC), ECM deposition is restricted to the colonic mucosal and submucosal layers. In CD, fibrosis can involve the full thickness of the bowel and result in luminal narrowing. Intestinal fibrosis, however, displays significant variability among IBD patients suggesting that fibrosis susceptibility may have a genetic component with conditioning by environmental and intestinal microbial factors(2).

Despite therapeutic advances in IBD, none prevent, nor reverse, established strictures(4). This implies that controlling inflammation may only partially affect fibrosis. The lack of any anti-fibrotic drugs is partly due to the main, and specific cellular and molecular fibrosis pathways remaining unidentified. New and improved preclinical animal fibrosis IBD models are thus needed(5). Anti-fibrotic drug development is also hindered by the unpredictable evolution of fibrosis as a clinical benefit may only be observed after prolonged treatment making clinical trials long and expensive.

To design effective anti-fibrotic drugs, fibrosis needs to be viewed as a pathological process distinct from inflammation. Understanding the mechanisms leading to intestinal fibrosis may thus pave the way for new anti-fibrotic agents(4). In this review, we describe the most important mechanisms that contribute to the initiation and progression of intestinal IBD fibrosis including the cellular and molecular mediators, the ECM molecules and matrix metalloproteinases(MMPs)/tissue inhibitors of metalloproteinases(TIMPs)-system, the

microbiota products, the role of fat, genetic and epigenetic factors, as well as the currently available experimental models.

#### **Key points**

- Intestinal fibrosis is common in IBD;
- Fibrosis is defined by excessive ECM;
- Fibrosis occurs as a consequence of inflammation and follows its distribution;
- Inflammation is necessary to trigger fibrosis, but may have a minor role in its progression;
- Therapeutic control of intestinal inflammation may not affect fibrogenesis;
- Fibrosis is a chronic and progressive process acting through complex cell/matrix/cytokine and growth factors interactions, but may be reversible;
- Understanding of the mechanisms behind intestinal fibrosis may pave the way for new anti-fibrotic agents.

## **2. CELLULAR AND MOLECULAR MEDIATORS OF INTESTINAL FIBROSIS**

Intestinal fibrosis is no longer considered inevitable and irreversible(1,2). Myofibroblast activation is a common feature in fibrosis and research of the innate and adaptive immune responses in IBD participate in the differentiation/activation of myofibroblasts(2). The Th17-type immune response is proinflammatory/profibrotic. Th2-type immunity, defined by interleukin(IL)-4, -5 and -13 production is also fibrogenic with IL-13 being the dominant mediator. By contrast, Th1-type immunity expressing interferon- $\gamma$ (IFN- $\gamma$ ) has anti-fibrotic activity. A detailed overview of how the immune mediators affect fibrotic disease of other organs, including liver, lung, skin and kidney, has been recently published and shall not be covered further here(1).

While in other organs the source of ECM-producing myofibroblasts is restricted to a few cell types, in the intestine multiple cell types may become activated myofibroblasts(2,4). These cells derive not only from resident mesenchymal cells (fibroblasts, sub-epithelial myofibroblasts[SEMFs] and smooth muscle cells[SMCs]) but also from epithelial and endothelial cells (via epithelial [EMT]/endothelial-mesenchymal transition [EndoMT]), stellate cells, pericytes, and bone marrow stem cells(2,4) (Figure 1). ECM-producing cells are activated by paracrine signals, autocrine factors, and pathogen-associated molecular patterns (PAMPs) derived from microorganisms that interact with pattern recognition receptors (PRRs) such as toll-like receptors(TLRs)(2). Myofibroblasts are also activated by products derived from injured cells, the ‘so-called’ damage-associated molecular patterns(DAMPs)

including DNA, RNA, ATP, high-mobility group box proteins(HMGB), microvesicles, and fragments of ECM molecules(2).

All intestinal ECM-producing cells act synergistically and are controlled by numerous molecular mediators(1,4) (Table 1). The most important include transforming growth factor- $\beta$  (TGF- $\beta$ ), activins, connective tissue growth factor(CTGF), platelet-derived growth factor (PDGF), insulin-like growth factor(IGF-1&2), epidermal growth factor(EGF), endothelin(ET-1, -2, -3), various cytokines, products of oxidative stress, components of the renin-angiotensin system(RAS), angiogenic factors(e.g. vascular endothelial growth factor-VEGF) and mammalian target of rapamycin(mTOR)(4). Soluble factors with anti-fibrotic properties have also been identified including peroxisome proliferator activated receptors(PPARs), IFN- $\alpha$ , IFN- $\gamma$ , IL-7, IL-10, IL-12, Smad7, adiponectin and nitric oxide(NO).

Although the TGF- $\beta$ /Smad pathway represents the major driving force of fibrosis, several pro-fibrogenic and anti-fibrogenic molecules seem to interact directly with the TGF- $\beta$ /Smad pathway (Figure 2). The effect of these mediators on the TGF- $\beta$ /Smad 'core pathway' have been extensively discussed in a recent review(4). The pharmacological modulation of ECM deposition by reducing ECM-producing cell activation and/or by modulation of specific molecular mediators could thus modify intestinal fibrosis(1,2,4).

Blockade of TGF $\beta$  signalling, either at the extracellular, or intracellular, level offers a strategy to prevent/treat fibrosis(1,4). Since TGF $\beta$ , however, is also involved in cellular differentiation, proliferation, transformation and immunoregulation, its blockade is problematic as TGF $\beta$ , Smad2 and Smad4 disruptions are lethal. Targeting of individual intracellular mediators, however, could lead to the selective blockade of TGF $\beta$  fibrotic responses without involving physiologically vital TGF $\beta$  responses(Figure 3). Disrupting Smad3 results in mice that survive to adulthood and also confers resistance to tissue fibrosis(4). Hepatic growth factor(HGF), bone morphogenetic protein(BMP-7) and decorin are three natural inhibitors of the TGF $\beta$ /Smad pathway and demonstrate anti-fibrotic effects.

In addition to the most common pro-fibrotic mediators including several growth factors, cytokines, chemokines and reactive oxygen species(ROS), new inducing and modulators factors of fibrosis are emerging (Table1). These include RAS, integrins, mTOR, PPARs, PAMPS, TLRs, DAMPs, ECM fragments, Hedgehog (Hh) signalling and Wnt/ $\beta$ -catenin and

Klotho pathways, receptor for advanced glycation endproducts (RAGE), Notch signalling pathway, microRNAs (miRNAs), as well as endoplasmic reticulum (ER) stress and telomere shortening.

### **2.1 Renin-angiotensin system (RAS)**

The RAS regulates cell growth, differentiation, proliferation and apoptosis, ROS generation, cytokine expression, endothelial cell activation, inflammation, ECM production and fibrosis(6). All components of the RAS exist in the intestine. Angiotensin II(ANGII), the principal effector of RAS, participates in fibrosis through the regulation of the inflammatory/fibrotic processes. ANGII is increased in CD intestine. Intestinal fibrosis is significantly improved, or even reversed, by Angiotensin converting enzyme(ACE) inhibitors and AT1 receptor antagonists, findings that closely correlate to reduced TGF- $\beta$ 1 and CTGF expression(7).

### **2.2 Integrins**

Integrins regulate cell and ECM interactions and influence fibrosis(4). In normal conditions  $\alpha$ v $\beta$ 6 integrin is not expressed, but is up-regulated and co-localises with TGF- $\beta$  following tissue injury(8).  $\alpha$ v $\beta$ 6 activates latent TGF- $\beta$ , while various genetic and pharmacologic interventions targeting it reduce TGF- $\beta$ 1 activation and fibrosis(9). Inhibitors of  $\alpha$ v $\beta$ 6 significantly reduce tissue levels of pro-fibrogenic transcripts, including procollagen  $\alpha$ 1,  $\alpha$ -SMA, TGF- $\beta$ 1, TGF- $\beta$ 2, CTGF, TIMP-1 and  $\alpha$ v $\beta$ 6 itself. Inhibition  $\alpha$ v $\beta$ 6 integrin could thus impact fibrosis, through local TGF- $\beta$  inhibition without affecting vital homeostatic TGF $\beta$  roles(4).

### **2.3 Mammalian target of rapamycin(mTOR)**

mTOR, a phosphatidylinositol 3-kinase-related kinase, forms at least two distinct complexes(10). The mTOR complex 1(mTORC1) is composed of mTOR, G $\beta$ L and Raptor and controls protein synthesis, cell growth/proliferation, autophagy, angiogenesis and fibrosis. The mTOR complex 2(mTORC2) consists of mTOR, G $\beta$ L and Rictor and is involved in cell proliferation and survival, metabolic regulation and actin cytoskeleton organization. mTOR signalling is activated by hormones, growth factors, amino acids, stress and alterations in cellular energy status. mTOR inhibitors(mTORis) have anti-fibrotic activities, reduce fibroblast/myofibroblast numbers and down-regulate pro-fibrogenic cytokine production.



Defective autophagy and angiogenesis may cause fibrosis. Control of angiogenesis and lymphangiogenesis, might improve fibrosis, particularly due to the connection between vascular remodelling and fibrogenesis in chronic inflammation(11). VEGF expression is increased in IBD and its blockage reduces fibrosis in animal models.

mTOR regulates hypoxia-inducible factor(HIF)1- $\alpha$ , VEGF and angiopoietin-2 expression, the main driving factors of neo-angiogenesis. TGF $\beta$ /Smad3 also activates mTORC1 and promotes collagen production by increasing HIF-1 $\alpha$  and mTORC1 inhibition prevents TGF- $\beta$ -induced HIF expression and fibrosis(12). The combined immunosuppressive and anti-fibrotic action of mTOR inhibitors like rapamycin, and its analogues, sirolimus and everolimus, are thus potential treatments in CD fibrosis.

#### ***2.4 Peroxisome proliferator activator receptors(PPARs)***

PPARs are nuclear receptors which regulate gene transcription(13). PPAR- $\gamma$  is present in colorectal mucosa and in adipocytes, monocytes, macrophages, dendritic, B and T cells. It modulates adipocyte differentiation, glucose homeostasis, lipid metabolism, inflammatory/immune processes and fibrosis. PPAR- $\gamma$  activation strongly correlates with the TGF- $\beta$ /Smad pathway as it directly antagonises Smad3, or down-regulates CTGF expression (Figure 2)(14). It also prevents the increase in profibrotic MMP-2, MMP-9, TIMP-1, PDGF and leptin activities(15,16). Reduced PPARs expression induces increased collagen production and fibrosis progression, whereas its overexpression prevents fibrosis(17). PPAR- $\gamma$  has received particular attention in recent years as its activation, both by natural and synthetic agonists, attenuates fibrosis in several organs including the intestine, and these anti-fibrotic effects are abolished by PPAR- $\gamma$  selective antagonists(13,18). PPAR- $\gamma$  is thus an innate protector against excessive fibrogenesis and a potential anti-fibrotic target in IBD.

#### ***2.5 PAMPs and TLRs***

Many fibrotic disorders have an infectious aetiology(19). In IBD, luminal bacteria are pathogenic and express PAMPs that activate immune and non-immune cells. PAMPs include lipopolysaccharide (LPS), bacterial DNA, and double-stranded RNA, and these bind to PRRs like TLRs(2).

TLRs act as microbial product sensors that trigger host defense and activate immune and both pro-inflammatory and pro-fibrotic gene expression. TLR activation is pivotal in IBD, suggesting that TLR over-expression could underlie abnormal host reactions to commensal

bacteria in IBD. CD patients with antibodies directed against microbial peptides develop earlier fibrostenotic disease(20). This defective immune tolerance to commensal bacteria suggests that an aberrant innate immune response is involved in intestinal fibrogenesis.

TLR expression in non-immune cells is a key event leading to fibrosis. Increased expression of TLR2, -3, -4, -6 and -7 occurs in CD and promote fibroblast differentiation into myofibroblasts and ECM expression(21).

The major *NOD2* polymorphisms associated with CD produce defects in host defense against invading bacteria leading to persistent intracellular infection(20). *NOD2* is expressed by Paneth cells with a correlation between *NOD2* variants and down-regulation of mucosal  $\alpha$ -defensin. *NOD2* gene variants in CD with/without variants of TLRs (TLR4) or ATG16L1 have an increased risk of small bowel (SB) fibrostenosis(20) (Figure 4).

## **2.6 DAMPs**

Activation of immune and non-immune cells can occur by products from injured cells, the DAMPs that promote inflammation. DAMPS include a wide range of products (DNA, RNA, ATP, HMGB1, ECM fragments, interleukins etc) which cause sterile inflammation, but whether DAMPs promote fibrosis is unclear(2). One of the best-characterized DAMPs is the HMGB1. Ethyl pyruvate inhibits HMGB1 release and decreases inflammation, ameliorates colitis and reduces intestinal cytokine production in IL-10<sup>-/-</sup> mice, while HMGB1 activation of the induced RAGE promotes NF- $\kappa$ B and MAP kinase signalling, resulting in inflammation(22). The HMGB1/RAGE pathway regulates metabolism and autophagy in experimental colitis and HMGB proteins function as universal sentinels for nucleic acid-mediated innate immune responses(23).

## **2.7 ECM components**

ECM regulates inflammation, healing and fibrosis(1). Intestinal ECM acts as a reservoir for pro-fibrotic factors, cytokines and chemokines. TNF- $\alpha$ , TGF- $\beta$  and bFGF interact with various ECM moieties, while ECM fragments bind to, and activate, TLR2 and TLR4. Fibrin, collagen IV and laminin fragments, modulate cell migration and proliferation, while fibrin and fibronectin promote EMT. Hyaluronan is essential for TGF- $\beta$ -induced myofibroblast differentiation and induces pro-inflammatory/fibrotic cytokine expression and MMP secretion(24).

## **2.8 Hedgehog signalling**

The Hedgehog(Hh) signalling pathway regulates fibrosis and progenitor cell proliferation and differentiation. It is pro-fibrotic and promotes myofibroblast activation, EMT, MMP release, TGF- $\beta$ 1 and ECM production(25). Conversely, Hh signalling inhibition is potently anti-fibrotic in preclinical models of fibrosis(26).

## **2.9 Wnt/ $\beta$ -catenin**

The Wnt- $\beta$ -catenin signalling pathway regulates cell growth, tumourigenesis and is activated in fibrosis. Activation of Wnt- $\beta$ -catenin signalling promotes EMT and is required for TGF- $\beta$ -mediated fibrosis(27). Involvement of the  $\alpha$ 3 $\beta$ 1 integrin occurs in the crosstalk between TGF- $\beta$ 1 and Wnt signalling. TGF- $\beta$  stimulates canonical Wnt signalling in a p38-dependent manner by decreasing the expression of the Wnt antagonist Dickkopf-1(DKK-1). In addition, hypermethylation of DKK1 promoters lead to aberrant Wnt signalling representing a link between epigenetic alterations and Wnt signalling in fibrosis(28). Wnt signalling increases ECM synthesis, T cell transmigration and regulates MMP-2, -7 and -9. Inhibition of Wnt- $\beta$ -catenin signalling reverses fibrosis.

## **2.10 Klotho**

Klotho family of membrane proteins function as obligate co-receptors for some fibroblast growth factors(FGFs)(29). The extracellular domain of Klotho protein is subject to ectodomain shedding and is released into circulation to act as an endocrine factor. Unlike membrane Klotho, which functions as a co-receptor for FGF23 to modulate FGF23 signal transduction, soluble Klotho is a multifunction protein present in biological fluids and impacts aging, energy metabolism, Wnt signaling inhibition, anti-oxidation, ion transport, RAS antagonism and fibrosis inhibition(30).

Secreted Klotho protein directly binds to the type-II TGF- $\beta$  receptor and inhibits TGF- $\beta$ 1 receptor binding, TGF- $\beta$ 1 signalling and TGF- $\beta$ 1-induced EMT. In addition, secreted Klotho inhibits the Wnt and IGF-1 signalling that promotes EMT. Overexpression of Klotho abolished TGF- $\beta$ 1-induced fibrosis(30).

## **2.11 RAGE**

This receptor for advanced glycation end products(AGEs) is fibrogenic, promotes EMT and ECM accumulation, increases activated myofibroblast numbers and up-regulates  $\alpha$ SMA(31). In IBD, high RAGE expression correlates with disease activity and induces pro-inflammatory

mediators(32). Ligands inhibiting RAGE, by competing with AGE, could be therapeutic in IBD fibrosis.

### **2.12 Notch signalling**

The Notch signalling pathway is essential to normal embryonic development, cellular proliferation and differentiation(33). Four Notch receptors and five ligands have been identified in mammals. Notch signalling is activated through the interaction of a Notch receptor with a ligand expressed on adjacent cells. The aberrant activation of this pathway induces fibrosis(34). Blocking Notch signalling with c-secretase inhibitors significantly attenuates fibrosis and decreases snail, vimentin, TGF- $\beta$ 1 and EMT(34).

Notch signalling, particularly Notch1, cooperates with TGF- $\beta$  in regulating FoxP3 expression during T regulatory cell (Treg) generation. Tregs are linked to fibrosis amelioration, although its specific role in fibrogenesis is less clear: it is plausible that Tregs could suppress Th17- and Th 2-driven fibrosis(35).

### **2.13 miRNAs**

miRNAs are small, noncoding RNAs of 18-25 nucleotides that regulate gene and protein expression by repressing specific target genes post-transcriptionally. Over 80 miRNAs are implicated in fibrosis by modulating ECM remodeling, cell adhesion, inflammation, angiogenesis and EMT(36). miRNA-21 and miRNA-29 promotes fibrosis by regulating TGF- $\beta$ 1/Smad and MAP kinase signalling, as well as CTGF and collagen expression. miRNA-192, miRNA-216a and miRNA-217, as miRNA-21, are key triggers of TGF- $\beta$  and Smad3-driven fibrosis and miRNA-200a and miRNA-200b are involved in CD fibrosis(37).

Some miRNAs like miRNA let-7d, miRNA-133, miRNA-30, miRNA-150, miRNA-194, and miRNA-200a are constitutively expressed in healthy tissues but down-regulate in fibrosis, suggesting an anti-fibrotic role. Specific miRNAs down-regulate Smad-3 activity and ECM expression, and prevent TGF $\beta$ -dependent EMT. miRNA-200 regulates TGF- $\beta$ /Smad-induced EMT by controlling the Zinc finger E-box-binding homeobox 1 and 2 (ZEB1 and 2)(38). The miRNA changes in IBD and their specific role in fibrosis deserve further and more exhaustive investigations.

### **2.14 Endoplasmic reticulum (ER) stress**

ER stress leading to apoptosis of key structural cells may regulate fibrosis(39). Excess accumulation of unfolded, or misfolded proteins in the ER activates cellular stress pathways.

ER stress is common in IBD(40). Genetic and environmental factors affect intestinal ER stress and inflammation(40). Genetic factors include either primary ER Stress (XBP1, AGR2, ORMDL3) or secondary ER stress (HLAB27, Mucins, ATG16L1). Environmental factors include bacteria, dietary and drugs. Induction of ER stress activates several EMT-related pathways, including TGF- $\beta$ /Smads, Wnt/ $\beta$ -catenin, and Src(41). A better understanding of the mechanisms of ER stress could help in fibrosis prevention.

### ***2.15 Telomere shortening***

Telomere shortening impacts on pulmonary fibrosis and occurs with mutations in gene encoding telomerase reverse transcriptase, an enzyme crucial to the maintenance of telomere length(1). Pro-fibrotic mediators, like TGF- $\beta$ 1 and ROS, also participate in telomere shortening suggesting a vicious cycle among pro-fibrotic mediator production, impaired telomerase activity and fibrosis(42). Unfortunately, data on telomere shortening in IBD are lacking.

#### **Key points**

- Myofibroblasts derive from mesenchymal, epithelial, endothelial, and stellate cells, pericytes, and bone marrow stem cells;
- ECM-producing cells act synergistically and are under numerous biological mediator control;
- Blockage of selective signalling pathways can prevent/reverse intestinal fibrosis.

#### **Questions**

- What are the cellular triggers leading to fibrosis progression?
- What is the main source of myofibroblasts in intestinal fibrosis?
- What are the main mediators of myofibroblast activation?
- What are the specific molecular markers of activated myofibroblasts?
- Which factors determine the switch from inflammatory to fibrosing disease?
- Can timing, concentration and the source of the main pro-fibrotic mediators affect their contribution to tissue remodelling/fibrosis?
- Is the simultaneous action of pro-fibrotic mediators relevant to fibrogenesis?
- Which factors represent the driving force (“core pathway”) of intestinal fibrosis?
- Which factors with anti-fibrotic properties play a critical role in intestinal fibrosis?

## **3. ECM MOLECULES AND MMP/TIMP SYSTEM**

Fibrogenesis hinges on the balance between ECM deposition and degradation. If deposition outstrips degradation then fibrosis may occur, but if this balance can be modified then healing may proceed without fibrosis(3,43).

### **3.1 Extracellular Matrix(ECM)**

The intestinal ECM is comprised of structural proteins, particularly the collagens, specialized proteins, like vitronectin and fibronectin, and matricellular proteins, such as osteopontin and trombospondin(Table 2). Chronic intestinal inflammation leads to tissue damage with an increase in ECM turnover. ECM degradation is mediated by MMPs and the fine balance between MMPs and TIMPs appears to be disturbed in IBD(43). It is unclear, however, which specific MMPs and TIMPs are involved in fibrosis and how they are regulated.

### **3.2 MMPs/TIMPs System**

MMPs are zinc- and calcium-dependent ECM degrading endopeptidases and collectively can degrade all ECM proteins. In addition, MMPs can proteolytically activate, or degrade, a variety of non-matrix substrates, including chemokines, cytokines, growth factors, and junctional proteins, which are usually secreted in an inactive preform.

Depending on substrate specificity, amino acid similarity, and identifiable sequence modules, the MMPs can be classified into: collagenases, gelatinases, stromelysins, matrilysin, membrane-types and others(44) (Table 2). MMPs are regulated by two endogenous inhibitors,  $\alpha$ 2-macroglobulin and the TIMPs.

MMPs are up-regulated in colitis models and IBD(43). MMP-9 and -3 are associated with mucosal damage and fistulae in CD, MMP-1, -3, and -13 with intestinal ulcers and MMP-10 and -11 with epithelial dysfunction. In fibrosis, MMP-1 and collagen I, but not MMP-3 or collagen III, levels were elevated in the TNBS murine fibrosis model which parallel that in fibrosed CD(45). Mucosa overlying strictured fibrostenosing CD demonstrate higher TIMP-1 levels and lower Smad7, MMP-12 and -3 expression than from mucosa overlying non-strictured areas(46).

Soluble mediators that are present in inflamed IBD modulate MMP and TIMP expression(2,4). TGF- $\beta$ 1 increases collagen secretion, decreases MMP-1 and increases TIMP1 production resulting in a net increase in collagen deposition. PDGF and IGF-1 also increase collagen production, but do not alter TIMP-1. IGF-1 decreases MMP-1 while PDGF increases its production. By contrast, enhanced cyclooxygenase(COX)-2 activity can block the pro-fibrogenic effects of TGF- $\beta$ 1 in SMCs and inhibit basal, and stimulated, levels of TIMP while inducing MMP secretion by macrophages. Activation of COX-2 also increases

prostaglandin(PG)-E2 production and an increase in PGE2 decreases fibroblast and SMC proliferation.

Indomethacin, a non-specific COX inhibitor, induces intestinal inflammation and fibrosis in the TNBS mouse model(47). In this model PGE2 decreased intestinal inflammation and fibrosis and in PMA±Indomethacin-treated murine and human colonic fibroblasts, PGE2 significantly decreased ECM deposition by decreasing TGFβ, collagen and TIMP expression, while increasing MMP expression.

TIMPs inhibit MMPs(48). TIMP-1 and -3 are inducible, while TIMP-2 is constitutively expressed. A critical balance between MMPs and TIMPs maintains normal ECM homeostasis. An imbalance may enhance ECM deposition and fibrosis. Furthermore, TIMPs affect signalling and angiogenesis and TIMP-1 expression inversely correlates with pro-inflammatory cytokine production, mucosal injury and disease activity(48). Increased TIMP-1 levels occur in CD strictures(46).

TIMP-3 is tightly bound to the surrounding matrix, is detected in normal and inflamed intestine and is expressed below ulcers and in the LP surrounding damaged crypts. The ratio between MMP-1/TIMP-1 and MMP-3/TIMP-1 is increased in inflamed IBD tissue and MMP-3 levels are increased in fibroblasts and mononuclear macrophage-like cells with concomitant low levels of TIMP-1, TIMP-2 and TIMP-3 in intestinal CD fistulae(49). TGFβ1 down-regulates MMP and enhances TIMP-1 expression.

### ***Conclusion***

MMPs and TIMPs are central to ECM turnover and actively regulate inflammation and remodelling. The net effect, however, is not dependent only on the MMP/TIMP balance, as functional receptors that mediate downstream signalling of TIMPs may provide additional perspectives for consideration when examining the role of MMPs and TIMPs in fibrosis.

#### **Key points**

- ECM components are active players in fibrosis;
- MMP/TIMP balance alterations are observed in intestinal fibrosis;

#### **Questions**

- How do ECM components contribute to fibrosis?
- What are the main sources of MMPs and TIMPs?
- Which MMPs and TIMPs are most important?
- How do MMPs and TIMPs contribute to intestinal fibrosis?

## 4. MICROBIOTA PRODUCTS

The whole gut microbiome can be considered a true organ that is geared up for protecting our health and well-being and is amenable to modulation. Co-evolution of host and bacteria results in a symbiotic relationship, where the survival of harboured microbiota and human host is interdependent.

While the microbiota is part of the internal gut microenvironment, this organ is subject to external macroenvironmental influences. The established concept is that all environmental elements may influence disease, and several environmental factors can influence immune responses leading to fibrosis. Given the enormity and diversity of the external environment, new tools and approaches are needed to evaluate the impact of the “exposome” on intestinal disease and fibrosis(2).

The dominant mucosa-associated microbiota appears different from luminal faecal microbiota but is highly conserved in different intestinal segments. Bacteria residing in the mucous layer form a protective ‘living wallpaper’ against exogenous bacteria through colonisation resistance. Once the gastrointestinal mucus layer is disrupted, bacteria directly contact with epithelial, dendritic, lymphocytic and stromal cells and endanger mucosal homeostasis.

Chronic alteration of the intestinal mucosal barrier and microbiota may be critical in IBD pathogenesis, as well as in fibrosis(50,51). Molecular evaluation allows for the definition of “normal” microbiota, or normobiosis and exploring the dysbiosis in disease and its role in fibrosis.

### 4.1 Microbiota in IBD

Dysbiosis in IBD is characterized by increased bacterial density at the mucosal level, increased proportions of immuno-aggressive commensals, reduced proportions of anti-inflammatory commensals, and increased proportions of proteins that promote autoimmunity(51). Such dysbiosis creates a vicious circle favouring chronic inflammation. *F. prausnitzii* has anti-inflammatory properties which induces high IL10/IL12 cytokine release, reduces IL-1 $\beta$  and induces IL-8, and abolishes TNF- $\alpha$  induced NF- $\kappa$ B activity. The gut microbiota is involved in intestinal fibrogenesis by activating mesenchymal cells



through TLRs and NOD-like receptors, but not all bacterial products are pro-fibrogenic(2).

#### ***4.2 Genetic influences***

Since microbial composition is established early in life, host genetics may impact on bacterial composition and the immune responses to commensal bacteria and mucosal barrier function(52,53). The possible mechanisms for genetic regulation of enteric microbiota include altered Paneth cell function and expression of antimicrobial peptides, altered mucus production, altered secretion of IgA and IgM, and altered innate and adaptive immune responses.

Genes associated with CD include NOD2, that is linked to  $\alpha$ -defensin production and intracellular bacteria clearance, ATG16L1, that is involved in autophagy and phagocytosis of bacteria, neutrophil cytosolic factor 4 (NCF4) that is involved in NADPH-mediated killing of phagocytosed bacteria, immunity-related GTPase family M protein (IRGM), which impacts on IFN- $\gamma$ -induced killing of phagocytosed bacteria, and TLR4 that modulates the immune response toward bacteria. NOD2 polymorphisms, with/without TLR and ATG16L1 polymorphisms, increase SB fibrostenosis(20) (Figure 4). Patients with a stronger immune response to microbial peptides are also more likely to develop earlier complicated CD(20).

#### ***4.3 Microbiome effects***

The persistence of exogenous, and endogenous stimuli, provided by infectious pathogens are major promoters of fibrosis(19). Thus an important role in fibrosis for the gut microbiota is suggested(51). Unfortunately very few models can study intestinal fibrosis in conjunction with the microflora(5). Although patients with NOD2 variants have more fibrosis, the NOD2 knock-down animal models, or animals overexpressing a variant corresponding to the human mutations, do not develop fibrosis(20). By contrast the spontaneous SAMP1/YitFc mouse model a CD-like chronic ileitis demonstrates fibrosis and these are abrogated under germ-free conditions.

There is thus doubt to the role of the microbiome in fibrosis. One model used transplanted small intestine pieces into the neck fold of the same, or another, rat strain with TGF- $\beta$  and other fibrosis mediators rapidly up-regulated and fibrosis developing within three weeks(54). The same model in mice confirmed the data but no differences in fibrosis between WT and Myd88-deficient mice were found, indicating that at least, under the

artificial conditions of this model, innate immune signalling was not central to the fibrosis(54).

In liver fibrosis, impaired intestinal barrier function occurs with increased bacterial wall product translocation into the portal vein activating hepatic stellate cells(HSCs)(55). Cystic fibrosis also was influenced by bacterial stimuli as variants of the Cystic Fibrosis Transmembrane Resistance Protein (CFTR) were associated with faecal microbiota shifts, which aggravated the disease(56). These represent clear examples of microbial contribution to fibrosis.

### **Conclusion**

The IBD microbiota is different, and markers of microbiota are associated with complicated CD. It is unknown, however, if these differences are cause or effect. Nor is it known if they increase inflammation/fibrosis. It is thus important to study the relationship between fibrosis and IBD microbiota and determine if specific pro-fibrotic, or preventive microbial, compositions exist. This needs to be done knowing that there is huge variability in the human microbiome composition and that genetic risk factors for IBD may shape the microflora composition. Such studies will, therefore, need to be done in large cohorts of phenotypically well-characterized patients.

#### **Key points**

- Intestinal microbiota maintains intestinal mucosal barrier function but may also impair barrier function
- Intestinal microbiota may impact IBD pathogenesis/fibrosis;
- A dysbiosis occurs in IBD, which is different between CD and UC;
- Serum markers for microbiota are associated with fibrostenotic CD.

#### **Questions**

- The microbiota affects intestinal inflammation, but does it affect fibrosis independently of inflammation?
- Are there bacteria, or viruses, present in IBD that promote fibrosis without inflammation?
- How do microbial products impact on myofibroblast activation?

## **5. ADIPOSE TISSUE**

Adipose tissue is an energy regulator but also displays advanced endocrine and immunological properties. The latter occurs through its cellular composition that includes adipocytes, but also preadipocytes, macrophages, lymphocytes, fibroblasts and endothelial

cells. These cells generate a dense network of soluble factors that include adipokines, classical cytokines, chemokines, growth factors and hormones(57). A role for mesenteric adipose tissue(MAT), the white adipose tissue(WAT) in IBD intestinal fibrosis is recognised(58).

### ***5.1 Creeping Fat***

In IBD, mesenteric fat is altered and may present as ‘creeping fat’ or ‘fat wrapping’ while ‘mesenteric obesity’ can occur in CD(57). ‘Creeping fat’ extends from the mesentery and covers at least 50% of the bowel circumference. These changes occur in >50% of CD patients. It correlates with inflammation severity with the terminal ileum most frequently involved. More interestingly, abnormal collagen deposition and stricturing are common findings in the bowel with fat wrapping suggesting a relationship between adipose tissue and fibrosis.

### ***5.2 Adipose-tissue macrophages***

Adipose tissue demonstrates unique functional characteristics, which may contribute to inflammation and fibrosis in IBD. Adipose tissue macrophages (ATMs) and T lymphocytes (ATTs) from active IBD release more IL-6, IL-4 and IL-13 than inactive disease and controls(59). A clear increase in the proportion of ‘alternatively activated’ or M2 macrophages is present in creeping fat(60). M2 macrophages are involved in tissue repair and collagen deposition(1). ATMs are under the constant influence of locally produced soluble mediators including adipokines(60). The functional importance of ATMs and ATTs, and the potential association with fibrosis, however, remains unclear.

Pro-inflammatory mediators stimulate M1 macrophages, while M2 are polarised by Th2 cytokines. Epigenetic changes and non-coding RNAs are involved in this process, as miR-155 diminishes IL-13-induced M2 polarisation through reducing levels of IL-13. Mechanistic studies have demonstrated that M2 macrophages are not required for a Th2 response(1). Arg1-expressing M2 cells are required for the suppression and resolution of fibrosis as M2 cells compete with Th2 cells and fibroblasts for l-arginine, which is required for the production of l-proline and collagen(61). Rather than promoting fibrosis, M2 cells inhibit ECM synthesis and fibrosis thus their role in fibrosis requires further investigation.

### ***5.3 Adipocytes and microbial translocation***

As fibrosis may be triggered by bacterial-derived factors it is important that adipocytes and preadipocytes bear functional PPRs, which elicit an immune response to microorganisms. In

addition, adipocytes may transform into macrophages and exert direct anti-microbial activity. This suggests that fat wrapping is an anti-microbial defense mechanism that isolates affected bowel to contain intraluminal bacteria spread and prevent bacterial translocation in mesenteric adipocytes in CD(62). 'Creeping fat'-induced intestinal fibrosis could be a mechanism for preventing bacterial spread.

#### ***5.4 Adipocytes and Adipokines***

Adipocytes act as endocrine cells and, in IBD mesenteric adipose tissue and serum, secrete active pro- and anti-inflammatory molecules, known as adipocytokines or adipokines including leptin, adiponectin, resistin and ghrelin. Leptin is a cytokine-like protein that regulates immunity by promoting a Th1 profile, thus impacting CD pathogenesis(57,58).

Adiponectin has a similar structure to TNF- $\alpha$  and antagonizes TNF- $\alpha$  by competing for the same receptor and demonstrates anti-inflammatory activity. Adiponectin down-regulates intercellular adhesion molecule-1(ICAM-1), endothelial adhesion molecule-1(ECAM-1) and E-selectin, thus inhibiting inflammatory cell migration and also regulates wound healing and fibrosis(63). While leptin is profibrogenic, and liver fibrosis is decreased in leptin- or leptin receptor deficient mice, adiponectin is, by contrast, anti-fibrogenic as extensive liver fibrosis may develop in adiponectin-knockout mice and is alleviated by administration of recombinant adiponectin(63). A potential anti-fibrotic effect of visceral mesenteric fat could relate to adipocytokine autophagy regulation.

High levels of C1q/TNF-related protein-3(CTRP-3) expression occur in adipose tissue. CTRP3 is a paralog of adiponectin and a member of the CTRP superfamily. It is recognised as a novel adipokine widely expressed in CD(64). CTRP3 exerts anti-inflammatory/-fibrogenic effects in human colonic fibroblasts inhibiting TGF- $\beta$  release, reducing CTGF expression and collagen production.

#### ***5.5 Adipocytes and PPAR $\gamma$***

Mesenteric fat's role in intestinal fibrosis correlates with PPAR $\gamma$  activity(57,58). PPAR $\gamma$  affects inflammation/fibrosis in IBD as its activation in visceral mesenteric fat reduces TNF- $\alpha$  and leptin expression, while increasing adiponectin(65).

PPAR $\gamma$  activators are efficacious in a variety of experimental models of fibrosis. Specific synthetic ligands for PPAR $\gamma$  control fibrosis by down-regulating myofibroblasts proliferation

and migration, and inducing apoptosis, inhibiting profibrogenic TGF- $\beta$ , PDGF and leptin while up-regulating anti-fibrogenic HGF and adiponectin, and reducing ECM deposition(16,63). The inhibitory effects of adiponectin are mediated by AMP kinase activation. Moreover, genetic deletion of adiponectin in mouse fibroblasts abrogated the TGF- $\beta$  signalling inhibition by PPAR $\gamma$  agonists. Together, it suggests that the adiponectin/AMP kinase pathway is important in ECM regulation and fibrosis.

The importance of crosstalk between mesenteric fat and IBD intestine and its role in fibrogenesis is controversial. Further investigations may allow for analysis of mechanisms involved in fibrosis and point to potential anti-fibrotic strategies.

#### **Key points**

- ‘Creeping fat’ or ‘fat wrapping’ is specific for CD;
- The terminal ileum is most frequently affected by ‘fat wrapping’;
- Intestinal fibrosis is associated with ‘creeping fat’;
- Mesenteric adipose tissue contributes to pro-inflammatory/fibrotic responses.

#### **Questions**

- What are the adipose-tissue derived pro-inflammatory/fibrotic responses?
- What is the role of adipokines in fibrosis?
- Is there an adipose-tissue anti-fibrotic response?
- How do microbial factors elicit an immune and pro-fibrotic response from adipose tissue?
- Can ‘creeping fat’ represent an anti-microbial defense mechanism?

## **6. GENETIC AND EPIGENETIC FACTORS**

IBD fibrosis susceptibility may have genetic and/or epigenetic components. IBD Genome-wide association studies(GWAS) identified numerous genetic polymorphisms including single nucleotide polymorphism-SNP. In 2013 the number of IBD risk loci was 163, whose 110 are shared between CD and UC, 23 are specific for UC and 30 for CD(66). These polymorphisms impact the innate immunity, autophagy, intestinal barrier functions, IL-10 signalling and adaptive immunity. Some of these polymorphisms may promote the development of intestinal fibrosis.

These genetic variations, however, only account for approximately 20% of IBD cases. Other genetic mechanisms, therefore, may be involved, including heritable and reversible epigenetic alterations, notably DNA methylation and histone modification(66). In addition, miRNAs may affect IBD immunity and fibrosis(36). There is thus a need to comprehensively

characterize the functional genomic features of IBD and the environmental triggers together with specific disease phenotypes.

## **6.1 Genetics**

IBD fibrosis is dynamic and multifactorial and develops by interactions between genetic and environmental factors with different genetic polymorphisms influencing fibrosis in animal models and human case-control studies. These suggest that variants of genes encoding immunoregulatory proteins, pro- and anti-inflammatory cytokines, and fibrogenic factors may impact IBD fibrosis.

### ***NOD2***

The *NOD2* gene, on chromosome 16 was one of the first genetic candidate involved in intestinal fibrosis. The three variants of *NOD2* gene(R702W, G908R, L1007fsinsC) contribute as much as 30-50% of CD susceptibility.

There is a genotype/phenotype association between *NOD2* variants and fibrostenosing disease(20,67). A meta-analysis identified that a single *NOD2* variant increased the risk for intestinal fibrosis by 8% and up to 41% for 2 variants(67). The combination of *NOD2* variants and serological markers were associated with complicated disease(20). A large retrospective study of 1528 CD patients with >10yrs follow-up, detected increased probability of developing stenosing disease with *NOD2*, Janus kinase(JAK)2 and ATG16L1 mutations(68). Furthermore, CD patients carrying *NOD2* gene variants have an increased and early need for first surgery due to stricturing disease and higher rate of surgical recurrence(69).

The mechanisms that are responsible for the *NOD2*-induced fibrosis are unknown. This gene is important in intestinal autophagy against microbes and, in combination ATG16L1 and TLR variants, may contribute to the transcriptional and translational changes causing fibrosis(70). Interaction between different genes is also suggested with more fibrostenosing CD in individuals carrying the *NOD2* genotype and 4G/4G genotype of plasminogen activator inhibitor(PAI-I).

### ***ATG16L1***

Autophagy-related-16L1 gene(ATG16L1) variants(rs2241879 and rs2241880) favour fibrosis(68). Their impact, however, varies between patients and regions, thus ATG16L1 may be complementary to other gene mutations.

### ***TLRs***

TLR variants, especially TLR4, are associated with increased fibrostenotic SB CD(20,21). Multiple variants in TLR4 also result in liver fibrosis, whereas a TLR7 SNP protects from advanced liver inflammation and fibrosis(71).

### ***CX3CR1 gene***

Two polymorphisms(V249I and T280M) of chemokine fractalkine receptor CX3CR1 are associated with fibrostenotic CD, particularly in smokers and this is independent of NOD2(72,73).

### ***TGF- $\beta$***

TGF- $\beta$ 1 is the prototypical fibrogenic molecule, but although, TGF- $\beta$ 1 polymorphisms are associated with stricturing CD, and a shorter time to intestinal resection in adults(74), in children with IBD, intestinal fibrosis was not linked to four different TGF- $\beta$  mutations(75).

### ***Interleukin 23 receptor***

Variants in the interleukin 23 receptor(*IL23R*) gene are associated with CD fibrosis, with the TT genotype of *IL23R* rs1004819 variant associated with ileal and stricturing CD, but like ATG16L1 there are geographical differences(76).

### ***MMPs and TIMPs***

The balance between MMPs and TIMPs is vital in fibrosis. SNPs in genes encoding MMP-1, -2, -3, -9, TIMP-1 have been described in CD and the 5T5T genotype at the MMP-3 SNP-1613 5T/6T increases the chance of stenotic complications in CD(77).

## ***6.2 Epigenetics***

Epigenetics is the study of all heritable, and potentially reversible, genome functional changes that do not alter the nucleotide DNA sequence i.e. the regulation of gene expression(66). Epigenetics can be further defined as ‘the inheritance of variation above and beyond (epi)changes in the DNA sequence’ and represents mechanisms by which the environment may alter gene expression.

Various epigenetic mechanisms, including DNA methylation, histone modifications (histone methylation, acetylation, phosphorylation, sumoylation/ubiquitination) and formation of

particular chromatin structure, play crucial roles in the gene transcriptional expression in ECM-producing cells.

ECM degradation is also regulated through epigenetic modulation of matrix-associated enzymes. Epigenetic markers may thus be the missing link connecting the IBD internal micro- and external macro-environmental exposure to the transcriptome changes associated with fibrosis. The epigenetic control of inflammation and fibrosis, in IBD is not fully understood.

### ***DNA methylation***

Numerous intestinal disease-associated DNA-methylations occur in IBD(78). Changes in methylation states of IBD-associated genes are associated with gene expression shifts. Specific methylation may be associated with IBD where there are distinct DNA methylations profiles(1505 CpG sites of 807 genes). One study identified seven CsG sites where differential methylation occurred in IBD(78). Moreover, in IBD, subtype-specific changes in DNA methylation occurs, identified in several loci within the IL-12/IL-23 pathway(79).

It is unknown, however, how these methylation profiles impact on IBD genes. Demethylation of the MD-2 promotor site in the TLR4/MD-2 complex results in increased MD-2 expression, while IFN- $\gamma$  methylation levels correlate with the immune response to microbial components(80,81).

### ***Conclusion***

Genetic variations are associated with CD fibrosis, and most are associated with NOD2. Other mutations will, undoubtedly, be found, may clarify the complex characterisation and subsequent changes in functional genomics. Epigenetic studies in IBD, however, are in their infancy and future studies may potentially have high clinical utility, especially in the prediction of the future complication of intestinal fibrosis and the impact of therapeutic interventions.

#### **Key points**

- Genetic variants and IBD susceptibility are linked;
- NOD2 variants, alone or in combination with TLR or ATG16L1 polymorphisms are associated with fibrostenotic SB CD.
- Epigenetic modifications may amplify, or inhibit, the fibrogenic processes.

#### **Questions**

- Which gene variants alter the risk of IBD fibrosis and how?
- Which epigenetic modifications primarily affect fibrosis?
- How does the gut microenvironment and the external macroenvironment interact with the genetic/epigenetic factors associated with intestinal fibrosis?



## 7. ANIMAL MODELS

Animal models are essential for dissecting the pathogenetic mechanisms of inflammation-induced intestinal fibrosis. Understanding the similarities and differences between each model and human disease is essential as, currently, no single animal model truly recapitulates the chronic, fluctuating, progressive nature of IBD(5).

Animal models addressing intestinal fibrosis can be classified into seven categories: spontaneous, chemical-, bacterial-, immune- and radiation-induced, post-operative and gene knockout and transgenic models (Table 3). Besides the animal models, several *in vitro* systems are also available.

### 7.1 *Spontaneous Intestinal Fibrosis*

#### ***SAMP1/Yit mouse***

The SAMP1/Yit mouse is the most representative model of chronic intestinal inflammation, as disease develops spontaneously(82). Its similarities with CD include ileal lesions, segmental and transmural injury, granulomas and perianal lesions. Microbial factors accelerate, but are not essential, for the model. A striking feature is prominent hypertrophy of ileal muscularis propria with areas of focal fibrosis that result in segmental stricturing and pre-stenotic dilatation.

The mucosal immunophenotype of ileitis has two distinct phases(83). The induction phase is a typical, bacteria-related, Th1 response with no muscular hypertrophy or strictures. After week 10, the maintenance/chronic phase is established and is dominated by Th2/IL-13 responses(83). Alterations in ECM occur with collagen deposition and fibrostenosis. The SAMP1/Yit model offers unique opportunities to study induction and perpetuation, of inflammation-induced intestinal fibrosis.

### 7.2 *Chemically-Induced Models*

The ‘chemical’ models of intestinal inflammation/fibrosis are the most utilized because they are easy, reproducible and straightforward. They involve colonic injury induced by the local delivery of an offensive chemical and allow for the study of both inflammatory and mucosal repair mechanisms.

The mucosal events that follow a chemical insult are, however, not representative of the immunological phenomena in IBD and results should be analyzed with caution.

### ***TNBS***

TNBS-induced colitis is the best-characterized murine model of inflammation-induced intestinal fibrosis(45). Colorectal colonic fibrosis, lumen stenosis and bowel dilatation develops after several doses of TNBS. Fibrosis is associated with elevated mucosal pro-inflammatory/fibrogenic factor expression and morphological analysis of colonic mesenchymal cells reveal distinctive features and enhanced response to IFN- $\gamma$  stimulation, which primarily increase TIMP-1 expression(45).

Mucosal expression of IL-13 replaces the initial Th1-type response and induces TGF- $\beta$ 1 expression by week 8-9 post-TNBS administration. Abrogation of IL-13 signalling via soluble IL-13R $\alpha$ 2-Fc, or IL-13R $\alpha$ 2-specific siRNA prevents TGF- $\beta$ 1 upregulation and fibrosis(84). IGF-I and early growth response gene(Egr)-1 are also pivotal in this model.

Fibrosis depends on the presence of inflammation as it is effectively prevented by the prophylactic neutralization of the master inflammatory regulator NF- $\kappa$ B(45) and by the coadministration of retinoic acid, while enhanced by the co-administration of indomethacin(47).

### ***DSS***

Dextran sodium sulphate (DSS) is administered in the drinking water resulting in colonic injury followed by spontaneous healing. Colonic fibrosis occurs after a single, or repeated cycles, of DSS administration and is dependent upon the murine genetic background(85). After 5 days of DSS administration, collagen colonic deposition is evident and gradually increases with elevations of TGF- $\beta$ 1, MMP-2 and -9. This is associated with mucosal upregulation of IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-17a and IL-10 mRNA(86).

## ***7.3 Bacteria-Induced Models***

### ***Salmonella***

A post-Salmonella enteric serovar Typhimurium(*S typhimurium*) infection fibrosis murine model was reported and characterized by caecal inflammation and fibrosis(87). At the molecular level, TGF- $\beta$ 1 and its downstream mediator CTGF, and IGF-1 are upregulated in

regions with infecting microorganisms. A strong pro-inflammatory mucosal milieu, consisting of TNF- $\alpha$ , IFN- $\gamma$ , MCP-1, and IL-17 are also detected in infected ceca, providing a link between inflammation and fibrosis. This model demonstrates that inflammation precedes fibrosis and more importantly that limiting inflammation by early intervention limits fibrosis. When a certain pathological stage is reached, however, fibrosis is independent of inflammation and non-reversible. A serious drawback of this model is that *Salmonella* infection does not result in fibrosis in humans.

#### ***Adherent-invasive Escherichia coli***

Chronic adherent-invasive *Escherichia coli* infection in streptomycin-treated conventional mice induces an active T-helper 17 response, heightened levels of proinflammatory cytokines and fibrotic growth factors, with the development of transmural inflammation and fibrosis(88). Depletion of CD8+ T cells increases the caecal bacterial load and the level of inflammation and intestinal fibrosis in C57BL/6 mice, suggesting that they play a protective role. These findings suggest that chronic adherent-invasive *Escherichia coli* infection results in an immunopathology that is similar to that seen in CD.

#### ***PG-PS***

An intramural intestinal wall injection induces local inflammation, followed by bowel wall thickening and fibrosis with increased TGF- $\beta$ 1 and IGF-1 expression(89). Inflammation and fibrosis are triggered by products of the commensal intestinal flora and thus allows for the study of microbiota-induced fibrosis.

#### ***7.4 Immune-Mediated Models***

The T-cell transfer model is the prototypical immune-mediated model, where naive CD45RB<sup>high</sup> CD4+ T cells are injected into SCID mice resulting in severe transmural colonic inflammation and mild fibrosis(90). This model, however, has only been used to examine the immune-mediated events and not fibrosis. Since in IBD T cells are intimately involved in fibrosis, investigation of fibrogenesis in this model should be of use.

#### ***7.5 Radiation-Induced Intestinal Fibrosis***

Radiation can cause bowel inflammation and fibrosis, which is similar to CD with myofibroblast/SMC proliferation, vascular sclerosis and chronic ulcers(91). Endothelial cell dysfunction is key for radiation-induced intestinal fibrosis with prolonged upregulation of

fibrogenic cytokines in the irradiated bowel. Ras homologue (Rho) and Rho-associated kinase (ROCK) signalling pathways are involved, as are mast cells and their products.

## **7.6 Post-operative models of fibrosis**

Postoperative anastomotic fibrosis is common, but why it develops is unknown. In IL-10 KO mice following ileocecal resection, inflammation-driven small intestinal fibrosis occurs at the site of resection and adjacent SB(92). Interestingly, germ-free IL-10 KO mice don't develop fibrosis, suggesting a role for gut microbiota. As fibrosis occurs proximal to anastomosis, it represents an excellent model for the study of cellular and molecular mechanisms of this complication.

A new model is the heterotopic transplantation of SB resections in rats(54). Rapid loss of crypt structures occurs at day 2 after transplantation followed by lymphocyte infiltration and obliteration of the intestinal lumen by fibrosis at day 21, which is associated with increased expression of established mediators of fibrosis such as  $\alpha v\beta 6$  integrin, IL-13, and TGF- $\beta$ . Typical histologic and molecular features of fibrosis are observed in the heterotopic intestinal grafts, which suggests, that this new model could be instrumental in studying treatment of intestinal fibrosis.

## **7.7 Gene Knockout and Transgenic Models**

The development of intestinal fibrosis by the manipulation of selected genes represents a direct method for determining which specific immune abnormalities may lead to fibrosis. IL-10-deficient mice develop chronic enterocolitis(93). A typical Th1-dominant inflammatory cytokine appears early, whereas in late disease a Th2 profile emerges resulting in ECM accumulation. This model could be useful to investigate mucosal fibroblasts in fibrosis.

As TGF- $\beta$ /Smad signalling is important for fibrosis, disruption of TGF $\beta$ /Smad signalling reduces intestinal fibrosis(94). Forced intestinal overexpression of TGF $\beta$ 1 by an adenoviral vector results in inflammation/fibrosis, collagen deposition and myofibroblast infiltration with colonic obstruction in 50% of mice(95).

## **Conclusion**

Animal models are integral to basic and translational research. More importantly, novel anti-fibrotic therapies can be tested by directly manipulating specific molecular pathways. The inherent problems associated with such approaches, however, should be kept in mind:

1. No animal model truly represents human disease. Chemical models lack the chronic nature, while genetically manipulated models have single defects that do not recapitulate the polygenetic nature and generalized immune dysregulation. The few spontaneous models may thus offer the best opportunity for dissecting the fibrotic pathways.

2. As fibrosis primarily is a product of chronic inflammation, it is not easy to separate the anti-inflammatory from anti-fibrotic effects of treatments.

3. The majority of models demonstrate a dependence on TGF- $\beta$ 1 or IL-13 for fibrogenesis, but these are most probably complementary. It is not clear whether these are true biological phenomena or reflect current fibrosis-related research.

4. The natural history of each model needs to be considered. Different phases of inflammation should be recognized, their immunological characteristics identified, and temporal and aetiological association with fibrosis defined. For example, some models develop as typical Th1-mediated inflammatory disease but with chronicity shift towards a Th2/IL-13 predominant type where fibrosis occurs. It may thus be more appropriate that anti-fibrotic treatments aim at neutralizing the late, and not the early, responses. By contrast, the exact time-point at which fibrogenesis starts is not well defined and it may be much earlier than thought, suggesting that therapies used in parallel with anti-inflammatory treatment may be appropriate.

#### **Key points**

- There are few models of intestinal fibrosis;
- Most IBD animal studies have focused on immunity and inflammation;
- No single animal model recapitulates human IBD;
- Similarities and differences between the models and human IBD should be clearly delineated;
- Animal models represent indispensable tools;
- Findings in experimental models do not always translate into clinical practice.

#### **Questions**

- Is a standard definition for intestinal fibrosis in models required?
- Are animal models useful to identify pathogenic events of intestinal fibrosis and to test new therapeutics?
- Do results need to be replicated in different animal models?
- Are animal models useful to investigate early and late events of intestinal fibrosis?
- Which *in vitro* models are more useful and reliable to unravel the complex cellular and molecular mechanisms of intestinal fibrosis?

## **8. CONCLUSION**

Intestinal fibrosis is highly complex with the specific molecules determining the balance between physiologic repair and excessive ECM accumulation remaining unknown. Strong evidence indicates that inflammation triggers fibrosis, which, once established, may progress

independently. It is critical, therefore, to elucidate the cellular signals that promote fibrogenesis and act independently of inflammatory pathways and the immuno-inflammatory response. Defining the cellular and molecular mechanisms involved in intestinal fibrosis is key to the development of new therapies.

The concept of intestinal fibrosis has changed from being static and irreversible to a dynamic and reversible disease. Novel therapeutic strategies are under investigation to target specific steps in fibrogenesis with the aim of reducing, or reversing, IBD fibrosis. One hope is that researchers, funding agencies and pharmaceutical industries accelerate their efforts to identify, and develop, safe and effective anti-fibrotic therapies.

#### CONFLICT OF INTEREST

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Giovanni Latella has consulted to MSD Italy and Shire; has received research grants from Giuliani, Alfa Wassermann and Sofar; has received speaker's honoraria from AbbVie Italy and Chiesi.

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## FIGURE LEGENDS

Figure 1. Pathogenetic steps of intestinal fibrosis in IBD. ROS= reactive oxygen species; SMCs= smooth muscle cells; SEMFs= intestinal subepithelial myofibroblasts; ICC= interstitial cells of Cajal; EMT= epithelial-to-mesenchymal transition; EndoMT= endothelial-to-mesenchymal transition; ECM= extracellular matrix.

Figure 2. Relationship among several pro-fibrotic and anti-fibrotic mediators in the development of fibrosis. IL-13=Interleukin-13; CCL2=monocyte chemoattractant protein-1 (MCP1); CCL3=macrophage inflammatory protein-1 (MIP1); TGF- $\beta$ =transforming growth factor- $\beta$ ; CTGF=connective tissue growth factor; PDGF=platelet derived growth factor; IGF-I=insulin-like growth factors I; TLR-2,-4= Toll-like receptor-2,-4; miRNA=microRNA; EGF=epidermal growth factor; bFGF=basic fibroblast growth factor; ETs=endothelins; ACE=angiotensin converting enzyme; AT-II=angiotensin-II; mTOR=mammalian target of rapamycin; PPAR- $\gamma$ = peroxisome proliferator activator receptor- $\gamma$ ; INF- $\alpha$ & $\beta$ =interferon- $\alpha$ & $\beta$ ; HGF=hepatic growth factor; ECM= extracellular matrix.

Figure 3. Interaction between profibrotic transforming growth factor- $\beta$ (TGF- $\beta$ ) and anti-fibrotic peroxisome proliferator activator receptor- $\gamma$ (PPAR- $\gamma$ ) factors: need to identify and selectively modulate transduction and transcription signalings of ECM synthesis/degradation without to affect other physiological pathways.

Figure 4. Genetic variants and immune responses in Crohn's disease patients may predict the risk for fibrostenosis phenotype and surgery. NOD2=nucleotide oligomerization domain 2; TLRs=toll-like receptors; ATG16L1= autophagy-related-16L1 gene; Abs= antibodies; Ags=antigens; ASCA=anti-Saccharomyces cerevisiae antibodies; I2=pseudomonas-associated sequence I2; OmpC=outer membrane porin C of Escherichia coli; CBir1= bacterial flagellin cBir1.

**Table 1. Molecules involved in intestinal fibrosis**

<b>FIBROGENIC</b>	<b>ANTI-FIBROGENIC</b>
<ul style="list-style-type: none"> <li>-Transforming growth factor-<math>\beta</math> (TGF- <math>\beta</math>)</li> <li>-Smad2/3 proteins</li> <li>-Activin A</li> <li>-Connective tissue growth factor (<i>CTGF</i>)</li> <li>-Platelet derived growth factor (<i>PDGF</i>)</li> <li>-Insulin-like growth factors (<i>IGF-I and II</i>)</li> <li>-Epidermal growth factor (<i>EGF</i>)</li> <li>-Basic fibroblast growth factor (<i>bFGF</i>)</li> <li>-Cytokines (<i>IL-1<math>\beta</math>, IL-4, IL-6, IL-13, IL-17, IL-21, IL-22, IL-23, IL-33, TNF-<math>\alpha</math></i>)</li> <li>-CC- and CXC-chemokines (<i>CCL2, CCL3, CCL4, CCL20</i>)</li> <li>-Reactive Oxygen Species (<i>ROS</i>)</li> <li>-Integrins (<i><math>\alpha</math>V<math>\beta</math>6, <math>\alpha</math>V<math>\beta</math>8</i>)</li> <li>-Mammalian Target Of Rapamycin (<i>mTOR</i>)</li> <li>-PAMPs and TLRs (<i>TLR2&amp;4 ligands</i>)</li> <li>-DAMPs (<i>DNA, RNA, ATP, HMGB1, uric acid, fragments of ECM</i>)</li> <li>-Hedgehog (Hh) signalling pathway</li> <li>-Wnt- <math>\beta</math>-catenin signalling pathway</li> <li>-Advanced glycation endproducts receptor (RAGE)</li> <li>- Notch signalling pathway</li> <li>- MicroRNAs (miRNAs)</li> <li>- Endoplasmic reticulum (ER) stress</li> <li>-Vascular endothelial growth factor (<i>VEGF</i>)</li> <li>-Endothelins (<i>ET-1</i>)</li> <li>-Angiotensin Converting Enzyme (<i>ACE</i>)</li> <li>-Angiotensin-II (<i>AT-II</i>)</li> <li>-Norepinephrine</li> <li>-Thrombospondin-1,2</li> <li>-Leptin</li> <li>-Tissue Inhibitor of Metalloproteinases (<i>TIMPs</i>)</li> </ul>	<ul style="list-style-type: none"> <li>-Peroxisome Proliferator Activator Receptor-<math>\gamma</math> (<i>PPAR-<math>\gamma</math></i>)</li> <li>-Interferon-<math>\alpha</math> (<i>INF-<math>\alpha</math></i>)</li> <li>-Interferon-<math>\gamma</math> (<i>INF-<math>\gamma</math></i>)</li> <li>-IL-7, IL-10, IL-12</li> <li>-Smad7 protein</li> <li>-PGE2</li> <li>-Hepatocyte growth factor (<i>HGF</i>)</li> <li>-Adiponectin</li> <li>-Klotho</li> <li>-Nitric Oxide (<i>NO</i>)</li> <li>-Relaxin</li> <li>-Matrix Metalloproteinases (<i>MMPs</i>)</li> </ul>

**Table 2. ECM molecules and MMP/TIM-system in intestinal fibrosis**

<b>ECM MOLECULES</b>	
Collagens	<ul style="list-style-type: none"> <li>• Fibrillar Type Collagens I - III - V</li> <li>• Non-Fibrillar Collagen Type IV</li> </ul>
Glycoproteins	<ul style="list-style-type: none"> <li>• Laminin</li> <li>• Entactin/nidogen</li> <li>• Fibronectin/vitronectin</li> <li>• Tenascin</li> <li>• Sparc/BM40</li> <li>• Thrombospondin/osteopontin</li> </ul>
Proteoglycans	<ul style="list-style-type: none"> <li>• Glycosaminoglycans (Hyaluronic Acid)</li> <li>• Heparan Sulfate</li> <li>• Chondroitin Sulfate</li> <li>• Perlecan</li> </ul>
<b>PROTEINS MODIFYING ECM</b>	
Matrix Metalloproteinases	<ul style="list-style-type: none"> <li>• <i>Collagenases (MMP-1, -8, -13, -18)</i></li> <li>• <i>Gelatinases (MMP-2, -9)</i></li> <li>• <i>Stromelysins (MMP-3, 10, -11)</i></li> <li>• <i>Matrilysin (MMP-7)</i></li> <li>• <i>Elastase (MMP-12)</i></li> <li>• <i>Membrane types (MT-1,-2,-3,-4,-5,- 6-MM; namely MMP-14, MMP-15, MMP-16, MMP17, MMP-24, MMP-25, respectively)</i></li> <li>• <i>Others (MMP-19, -20, -21, -22, -23, 26, -27, -28)</i></li> </ul>
Tissue inhibitor of Metalloproteinases	<ul style="list-style-type: none"> <li>• <i>TIMP-1, -2, -3, -4</i></li> </ul>



**Table 3. Experimental Models to study IBD Fibrosis**

ANIMAL MODELS	CELL CULTURE SYSTEMS
Spontaneous <i>SAMP1/Yit mouse</i>	Cell lines cultures
Chemical-Induced <i>2, 4, 6-trinitrobenzenesulphonic acid (TNBS)</i> <i>Dextran sodium sulphate (DSS)</i>	Human cell primary cultures from IBD pts <i>Fibroblasts</i> <i>Myofibroblasts</i> <i>Smooth muscle cells</i> <i>Endothelial cells</i>
Bacteria-Induced <i>Salmonella</i> <i>Escherichia coli</i> <i>Peptidoglycan-polysaccharide (PG-PS)</i>	Microvesicles
Immune-Mediated	
Radiation-Induced	
Post-Operative	
Gene Knockout and Transgenic	

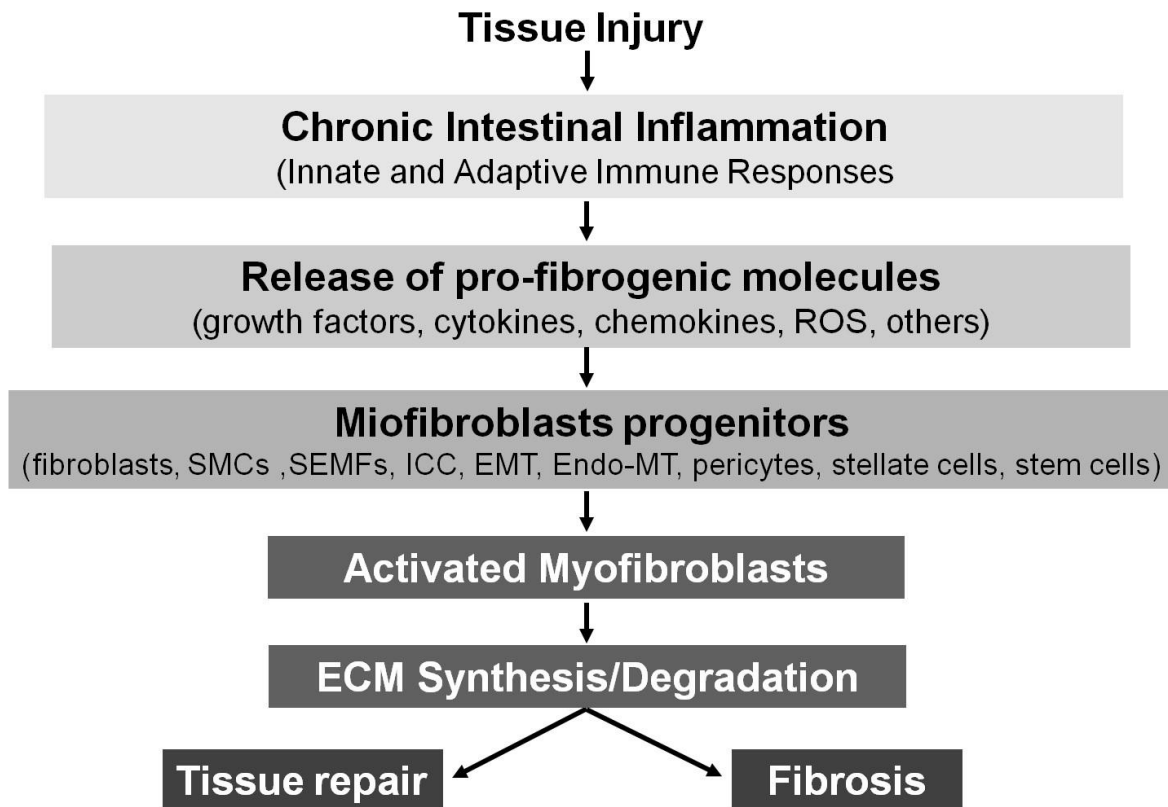


Figure 1. Pathogenetic steps of intestinal fibrosis in IBD. ROS= reactive oxygen species; SMCs= smooth muscle cells; SEMFs= intestinal subepithelial myofibroblasts; ICC= intestinal cells of Cajal; EMT= epithelial-to-mesenchymal transition; EndoMT= endothelial-to-mesenchymal transition; ECM= extracellular matrix.

## “Core pathway” of fibrosis.

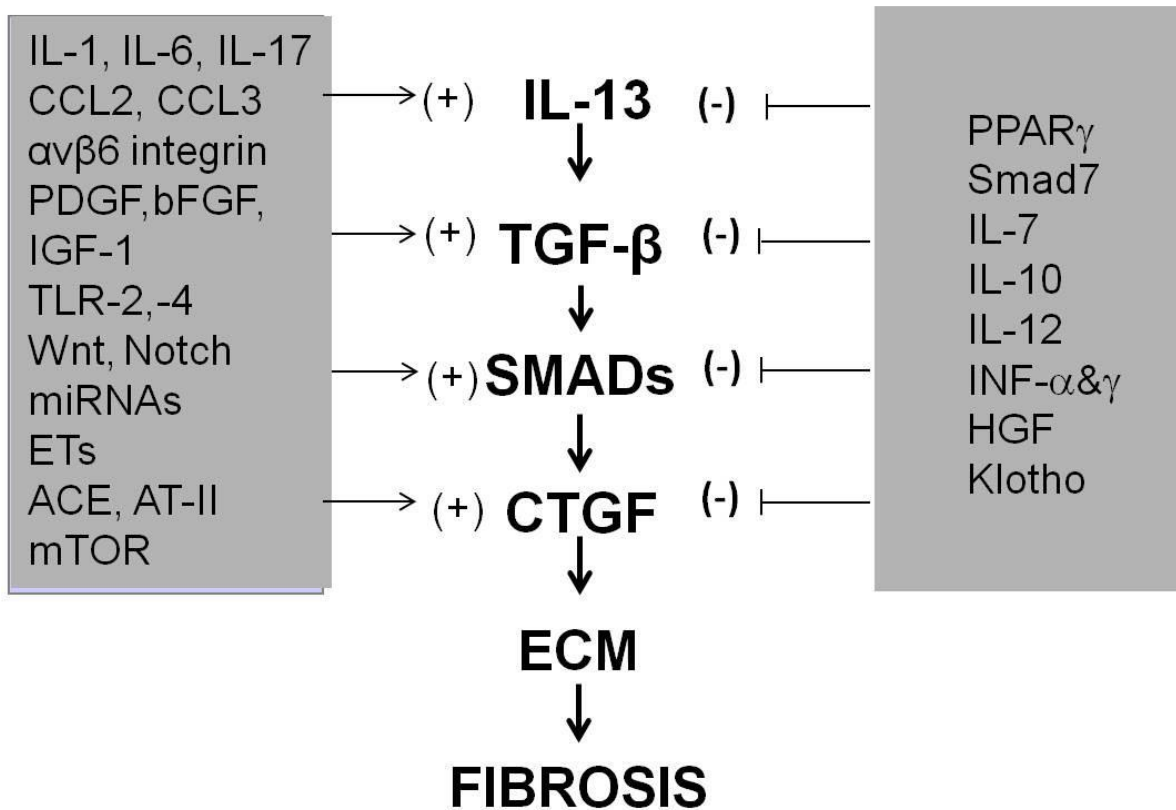


Figure 2. Relationship among several pro-fibrotic and anti-fibrotic mediators in the development of fibrosis. IL-13=Interleukin-13; CCL2=monocyte chemoattractant protein-1 (MCP1); CCL3=macrophage inflammatory protein-1 (MIP1); TGF- $\beta$ =transforming growth factor- $\beta$ ; CTGF=connective tissue growth factor; PDGF=platelet derived growth factor; IGF-I=insulin-like growth factors I; TLR-2,-4= Toll-like receptor-2,-4; miRNA=microRNA; EGF=epidermal growth factor; bFGF=basic fibroblast growth factor; ETs=endothelins; ACE=angiotensin converting enzyme; AT-II=angiotensin-II; mTOR=mammalian target of rapamycin; PPAR- $\gamma$ = peroxisome proliferator activator receptor- $\gamma$ ; INF- $\alpha$ & $\beta$ =interferon- $\alpha$ & $\beta$ ; HGF=hepatic growth factor; ECM= extracellular matrix.

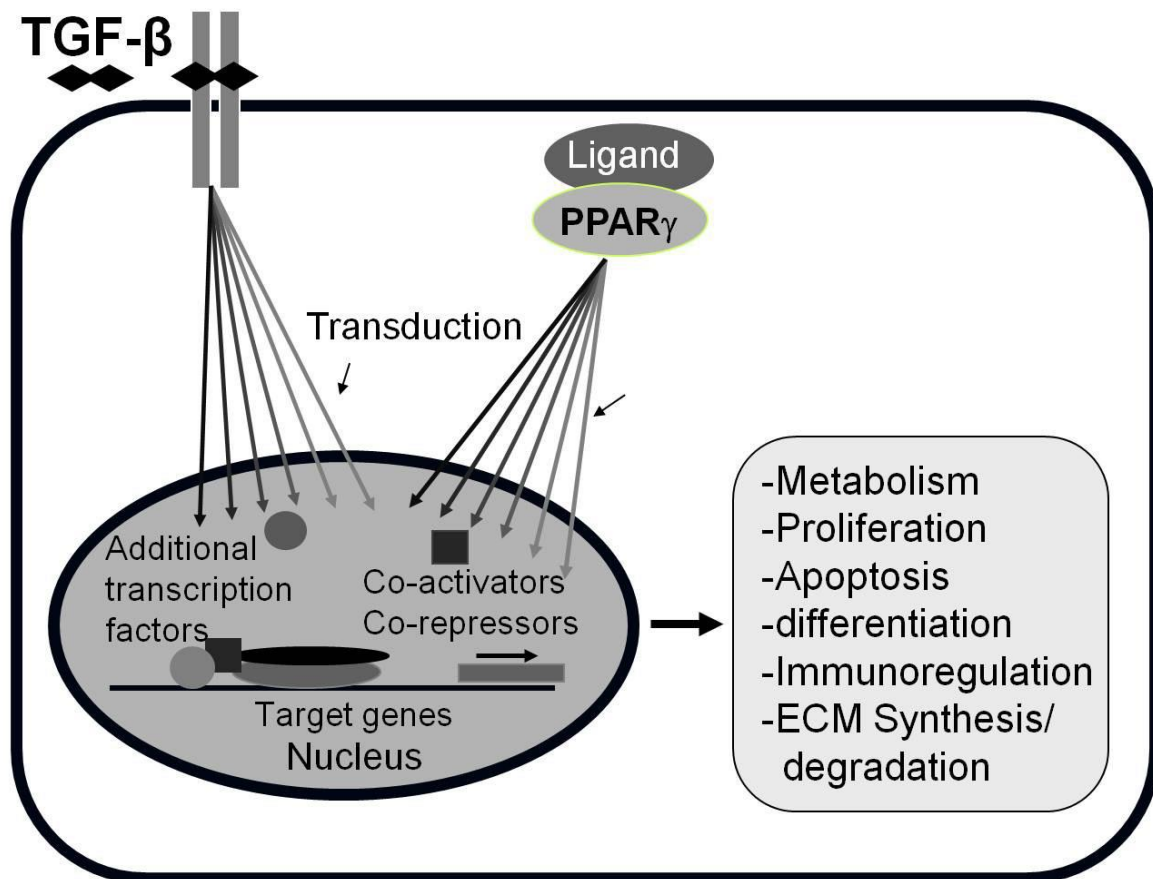


Figure 3. Interaction between profibrotic transforming growth factor- $\beta$ (TGF- $\beta$ ) and anti-fibrotic peroxisome proliferator activator receptor- $\gamma$ ( PPAR- $\gamma$ ) factors: need to identify and selectively modulate trasduction and transcription signalings of ECM synthesis/degratation without to affect other physiological pathways.

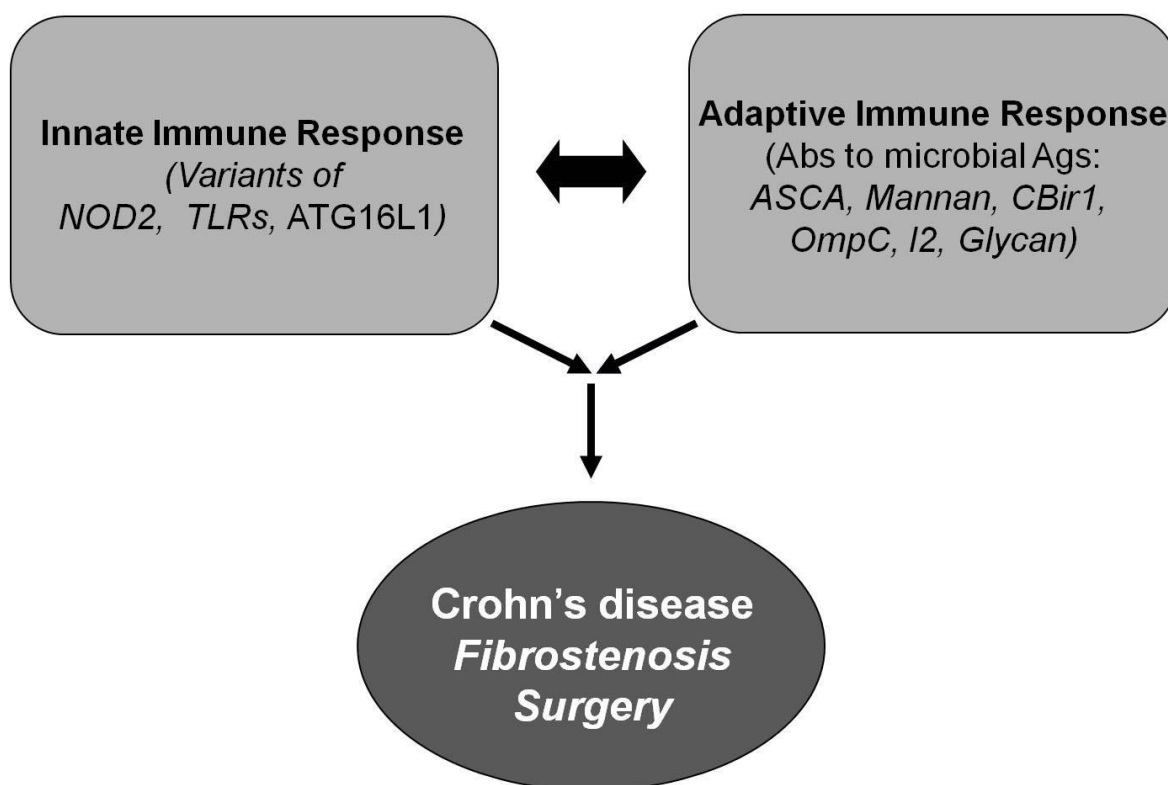


Figure 4. Genetic variants and immune responses in Crohn's disease patients may predict the risk for fibrostenosis phenotype and surgery. NOD2=nucleotide oligomerization domain 2; TLRs=toll-like receptors; ATG16L1= autophagy-related-16L1 gene; Abs= antibodies; Ags=antigens; ASCA=anti-Saccharomyces cerevisiae antibodies; I2=pseudomonas-associated sequence I2; OmpC=outer membrane porin C of Escherichia coli; CBir1= bacterial flaggelin cBir1.

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